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Decreased HSP70 expression on serum exosomes contributes to cardiac fibrosis during senescence

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Abstract. – OBJECTIVE: Aging is now considered as an independent risk factor for cardiac fibrosis. However, the mechanisms underlying aging-related cardiac fibrosis remain unknown. Here, we examine the role of serum exosomes in this process.

MATERIALS AND METHODS: Experiments were conducted using 6-week-old or 24-week-old male Sprague-Dawley (SD) rats. Cardiac sections were treated with Masson's trichrome stain to evaluate fibrosis. Exosomes were isolated from the serum, characterized and quantified using Western blot, electron microscopy, and qNano analysis, and co-cultured with transforming growth factor-β1 (TGF-β1)-induced primary cardiac fibroblasts (CF). Co-cultures were also carried out in the presence of a hot shock protein 70 (HSP70) inhibitor (gefitinib) or inducer (geranylgeranylacetone) to evaluate the role of HSP70 in cardiac fibrosis.

RESULTS: Cardiac fibrosis as well as serum exosomes levels were increased during senescence. We observed an increase in fibroblast proliferation and myofibroblast differentiation when CF were co-cultured with exosomes from old rats, compared to those from young mice. Observing a decrease in surface HSP70 expression on the exosomes derived from old rats, we tested the effects of HSP70 inhibition or overexpression on the CF co-cultures. HSP70 inhibition increased fibroblast proliferation and myofibroblast differentiation in CF co-cultures containing exosomes from the young rats, while HSP70 overexpression attenuated fibroblast proliferation and myofibroblast differentiation in CF co-cultures containing exosomes from the old rats.

CONCLUSIONS: Using an animal model of cardiac fibrosis, we show a decrease in HSP70 expression on the exosomal surface with aging, which may contribute to cardiac fibrosis.

Key Words
Senescence, Exosomes, HSP70.

Introduction

With the increase in human lifespan across populations, the prevalence of age-associated diseases such as heart failure or neurological disorders is also on the rise. Cardiovascular disease is the leading cause of death in developed countries. Data from the United States National Health and Nutrition Examination Survey examining trends between 2005 and 2008 showed that 80% of the cases of heart failure occurred in individuals over 65 years of age, suggesting that aging is a major risk factor for cardiovascular disease[1]. Furthermore, cardiovascular disease-associated morbidity and mortality also increases significantly with age. The increase in cardiovascular disease risk with age could be attributable to multiple factors, including a more prolonged exposure to various risk factors as well as intrinsic cardiovascular aging itself.

Aging is a multifactorial process characterized by a progressive loss of physiological integrity, leading to functional impairment and an increased risk of mortality[2]. Within the aging process, cellular and molecular changes associated with damage lead to increased disease susceptibility and mortality[3]. A key hallmark of the aging process is cellular senescence. During normal aging, the most common senescence-inducing factors include epigenetic, proteotoxic, and oxidative stress and telomere or DNA damage, whereas disease-related senescence has been linked to smoking and telomere or DNA damage[4]. Cardiac aging is a complex pathophysiological process accompanied by a number of biological changes, including cardiac remodeling and dysfunction[5]. Manifestations of aging-associated cardiac abnormalities include diastolic cardiac dysfunction, cardiac hypertrophy and fibrosis, and impaired contractile function[6]. Overall, cardiac fibrosis is a strong driver of

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adverse ventricular remodeling and heart failure. Characterized by excessive extracellular matrix accumulation, it is observed after various types of cardiac injuries, including myocardial infarction and conditions that cause hemodynamic stress, such as hypertrophic and dilated cardiomyopathy. While aging is a major risk factor for cardiac dysfunction, the relationship between cardiac fibrosis and aging remains unclear. Exosomes are small endosome-derived vesicles ranging from 30-200 nm in size that are actively secreted from cells through exocytosis, a process normally used for receptor discharge and intercellular cross-talk[7]. They are secreted by many cell types, including retinocytes, dendritic cells, B cells, T cells, mast cells, epithelial cells, and tumor cells[8-13]. Exosomes have been isolated and characterized from *in vitro* cultured cell lines, as well as in body fluids, including blood, urine, saliva, amniotic fluid, and malignant pleural effusions[14-19]. Exosomes have been shown to participate in the fibrotic process[20-22], but the relationship between exosomes and cardiac fibrosis in the context of aging is unknown. In this study, we examined changes in exosomes production during aging and investigated the effects of exosomes exposure on cardiac fibroblasts (CF), and studied the underlying mechanisms. This study shows that exosomes protect against cardiac fibrosis in an HSP70-dependent manner, and that increase in cardiac fibrosis with aging may be partly attributable to the aging-related decrease in HSP70 expression on exosomes.

Materials and Methods

Animal Studies

All animal experiments were approved by the Animal Research Committee of the Xinhua Hospital, and were in accordance with the principles established by the Guide for the Care and Use of Laboratory Animals (DHEW number NIH 86-23). Male Sprague-Dawley (SD) rats (either 6-month-old [denoted as 'young'] or 24-month-old [denoted as 'old']) were purchased from Shanghai Songlian Laboratory Animal Farms (production license SCXK2007-0011) and housed in a climate-controlled environment (22.8 ± 2.0°C at 45-50% humidity) with a 12/12-light/dark cycle, with free access to food and water. After allowing 1 week for acclimatization, blood and heart tissues were harvested following anaesthetization with 50 mg/kg sodium pentobarbital by intraperitoneal (i.p.) injection.

Masson's Trichrome Staining

Heart tissues were sectioned and dewaxed in the Histo-Clear reagent, washed three times with ethanol, and incubated with Masson's trichrome stain (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. The sections were then washed in acidified water and ethanol, and mounted in a resinous medium (Cytoseal, Hatfield, PA, USA).

Primary CF Isolation

CFs were prepared as previously described, using follow a conventional isolation method[23]. Briefly, heart tissues were dissected, minced, and enzymatically digested by incubation in 0.25% trypsin and 0.1% (*w/v*) collagenase II (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. Digestion was terminated by the addition of a double volume of HBSS (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% exosome-depleted fetal bovine serum (FBS; SBI, Palo Alto, CA, USA). Further mechanical disaggregation was achieved by pipetting. Tissue debris and cardiomyocytes were removed by sequential centrifugation at 100 rcf for 2 min, followed by passage through a 20-μm cell strainer, and centrifugation at 400 rcf for 5 min. Isolated fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% exosome-depleted FBS. Transforming growth factor-β1 (TGF-β1) (10 *μg/L,* Sigma-Aldrich, St. Louis, MO, USA) was added to stimulate fibroblast differentiation.

Exosome Preparation

Rats were anesthetized with 50 mg/kg sodium pentobarbital i.p. and placed on a heated mat. After thoracotomy, a 4-mL blood sample was rapidly removed into a citrated vacutainer™ to minimize platelet activation. Exosomes were prepared by standard differential centrifugation at 4°C as follows: centrifugation at 1,600 × g for 20 min to obtain plasma, and then 10,000 x g for 30 min to remove cells and platelets. Pellets were resuspended in phosphate-buffered saline (PBS) and centrifuged twice at 100,000 x g for 1 h at 4°C using a SW-41 rotor. For co-culture of exosomes with CF, plates were seeded with 1 x 10^5 CF/well, and exosomes were added at a final concentration of 40 μg/mL. Gefitinib (1 μM, WAKO Pure Chemicals, Chuo-ku, Osaka, Japan) and geranylgeranylacetone (1 μM; SigmaAldrich, St. Louis, MO, USA) were used to confirm the role of HSP70 in cardiac fibrosis. After 12 h incubation, the proliferation and differentiation of CF were measured.

Exosomes Characterization

Electron microscopy was carried out using the approach described by Malik et al[24]. Briefly, the pelleted exosomes were settled on a gold-coated grid, blotted, fixed in 1% glutaraldehyde, washed in purified water, and incubated with uranyl oxylate (5-10 min) and then with methylcellulose with uranyl acetate (5-10 min) three times. Following removal of methylcellulose-uranyl acetate, the exosomes were visualized by transmission electron microscopy using a Philips CM120 microscope (Amsterdam, The Netherlands). The whole-mounted exosomes were also immunostained with cmHSP70.1, and incubated with the secondary antibody, gold-labeled goat anti-mouse IgG, to visualize HSP70[25].

Exosomes quantification was done using qNano analysis (Izon Science, Burnside, Christchurch, New Zealand) based on the manufacturer's instructions. The protein levels in the exosomes isolate were quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

MTS Assay

CF proliferation was assessed by using the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit, Promega, Madison, WI, USA), based on the manufacturer's instructions. Fibroblasts were seeded in 96-well plates at an initial density of 5×10^3 cells/well. After allowing the cells to adhere for 2 h, exosomes were added and further incubated for 24 h. A curve of cell proliferation was constructed by measuring cell growth using a microplate reader at 490 nm.

Western Blotting

Western blotting for calnexin, CD9, CD63, and HSP70 was carried to verify that the surface marker expression was characteristic of exosomes[26]. Briefly, exosomal proteins were separated by electrophoresis (120 V for 50 min) on 10% sodium dodecyl sulfate/polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for 1.5 h at 100 mA. The following antibodies were used: anti-CD9, anti-CD63, anti-HSP70, and anti-calnexin (all from Abcam, Cambridge MA, USA). The membranes were washed three times in Tris-HCl with 0.1% Tween 20 for 5-10 min and incubated for 2 h in Tris-buffered saline and Tween-20 (TBST) containing a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Cambridge MA, USA). Proteins were then detected by enhanced chemiluminescence (Thermo Fisher, Waltham, MA, USA) and imaged by an ImageQuant LAS 4000 mini biomolecular imager (Bio-Rad, Hercules, CA, USA).

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. qRT-PCR was carried out using the Primer-Script one step RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) and the SYBR kit (TaKaRa), with detection on the ABI7500 system (Applied Biosystems, Foster City, CA, USA). mRNA levels were normalized against 18S RNA, and relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were used: alpha-smooth muscle actin (α-SMA) F: 5'-GTCCCAGACATCAGGGAGTAA-3′ and R: 5′-TCGGATACTTCAGCGTCAGGA-3′; collagen type 1 alpha 1 (Col1a1) F: 5′-GAGCGGAGAGTACTGGATCGA-3′ and R: 5′-CTGACCTGTCTCCATGTTGCA-3′; collagen type 3 alpha 1 (Col3a1) F: 5′-TGCCATTGCTGGAGTTGGA-3′ and R: 5′-GAAGACATGATCTCCTCAGTGTTGA-3′; connective tissue growth factor (CTGF) F: 5′-CACAGAGTGGAGCGCCTGTTC-3′ and R: 5′-GATGCACTTTTTGCCCTTCTTAATG-3′; 18S F: 5′-TCAAGAACGAAAGTCGGAGG-3′ and R: 5′-GGACATCTAAGGGCATCAC-3′.

Statistical Analysis

All values were expressed as the mean ± S.E.M. Student's *t*-test for unpaired results was used to evaluate differences between two groups. Differences were considered to be significant when $p < 0.05$.

Results

Cardiac Fibrosis is Increased During Senescence

Use of Masson's trichrome stain revealed increased fibrosis in the myocardium of old mice compared to young mice, especially in left atrium (Figure 1A). We also compared Col1a1, Col3a1, and CTGF mRNA levels in cardiac tissues and found significantly increased mRNA expression in the aging heart (Figure 1B).

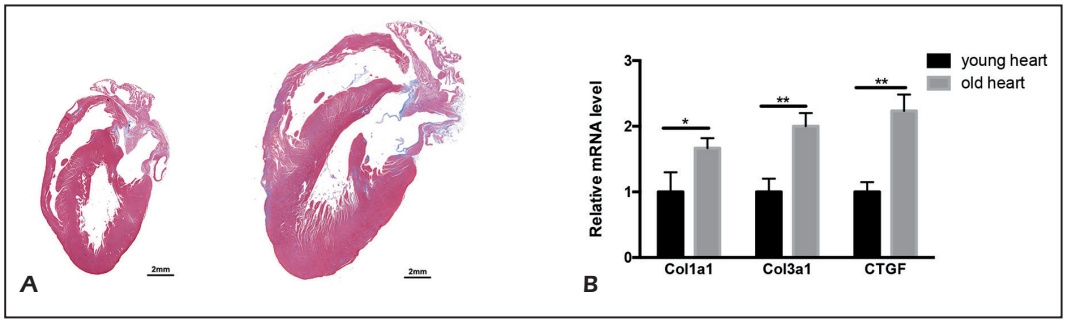

Figure 1. Cardiac fibrosis is increased during senescence. **A**, Masson stain of whole-heart sections from young (left) or old rats (right), showing increase in overall size as well as the level of fibrosis during senescence, especially in the left atrium. Scale bar, 2 mm. **B**, Comparison of Col1a1, Col3a1, and CTGF mRNA expression in heart tissue between young and old rats. *p<0.05, **p<0.01. Col1a1: collagen type 1 alpha 1; Col3a1: collagen type 3 alpha 1; CTGF: connective tissue growth factor.

Aging is Associated with Changes in Exosomes Expression

To examine whether exosomes expression was altered during senescence, we isolated serum exosomes from both groups of rats. Western blotting, electron microscopy, and nanoparticle tracking were used to confirm exosomes isolation. Western blotting verified that the surface marker expression was characteristic of exosomes, with enrichment of the exosomal markers CD63, CD9, and HSP70, and lack of expression of calnexin, an endoplasmic reticulum protein (Figure 2A).

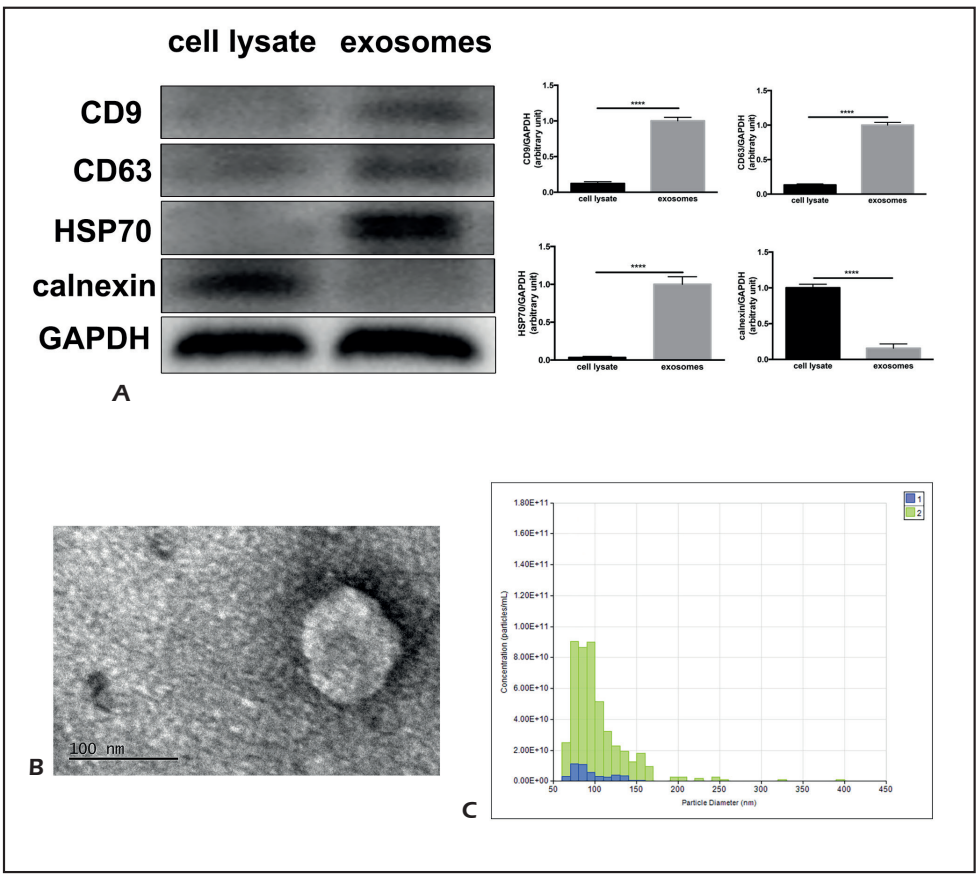

Figure 2. Characterization of serum exosomes. **A**, Western blotting of the cell lysate or exosome fractions for CD63, CD9, and HSP70, and calnexin. **B**, Electron microscopy of the isolated exosomes; bar, 100 nm. C, qNano analysis of exosomes; blue: exosomes from young rats; green: exosomes from old rats. ****p<0.0001.

Electron microscopy showed that the isolated vesicles, which appeared cup- or round-shaped, ranged between 30 to 200 nm in diameter (Figure 2B). Comparison of isolates from the plasma of young *vs.* aged animals by qNano analysis revealed that more exosomes were isolated from the serum of aged animals, suggesting that exosomes production increases during senescence (Figure 2C).

Exosomes from Old Rats Promote Fibroblast Proliferation and Myofibroblast Differentiation

To examine whether exosomes isolated from the old rats exerted any functional effect on cardiac fibrosis, we co-cultured TGF-β1-induced CFs with exosomes isolated from young or old rats. We found that compared to those from the young rats, exosomes from the aged rats promoted fibroblast proliferation and induced an increase in the expression of Col1a1, Col3a1, and CTGF (Figure 3A and B). Co-culture with exosomes from aged compared with younger rats also resulted in an increase in myofibroblast differentiation, as evidenced by an increased expression of α-SMA and TSP-1 (Figure 3C and D).

The Exosomes-Induced Effects on Cell Proliferation and Differentiation are Dependent on HSP70

We next examined whether HSP70, which is expressed on the exosomes surface, played a role in the exosomes-induced effects on CFs. Electron microscopy revealed that surface expression of HSP70 on exosomes decreased with aging (Figure 4A), which was confirmed by Western blotting (Figure 4B). Moreover, CF proliferation and myofibroblast differentiation were increased by co-culture of TGF-β1-induced CFs with exosomes derived from young rats in the presence of the HSP70 inhibitor, gefitinib (Figure 4C). Similarly, the increase in CF proliferation and myofibroblast differentiation induced by CF co-culture with exosomes from aged rats was attenuated by geranylgeranylacetone (GGA), which acts as an HSP70 inducer (Figure 4D).

Discussion

Advanced age has emerged as the strongest risk factor for cardiovascular disease, and the mechanisms underlying cardiac aging require

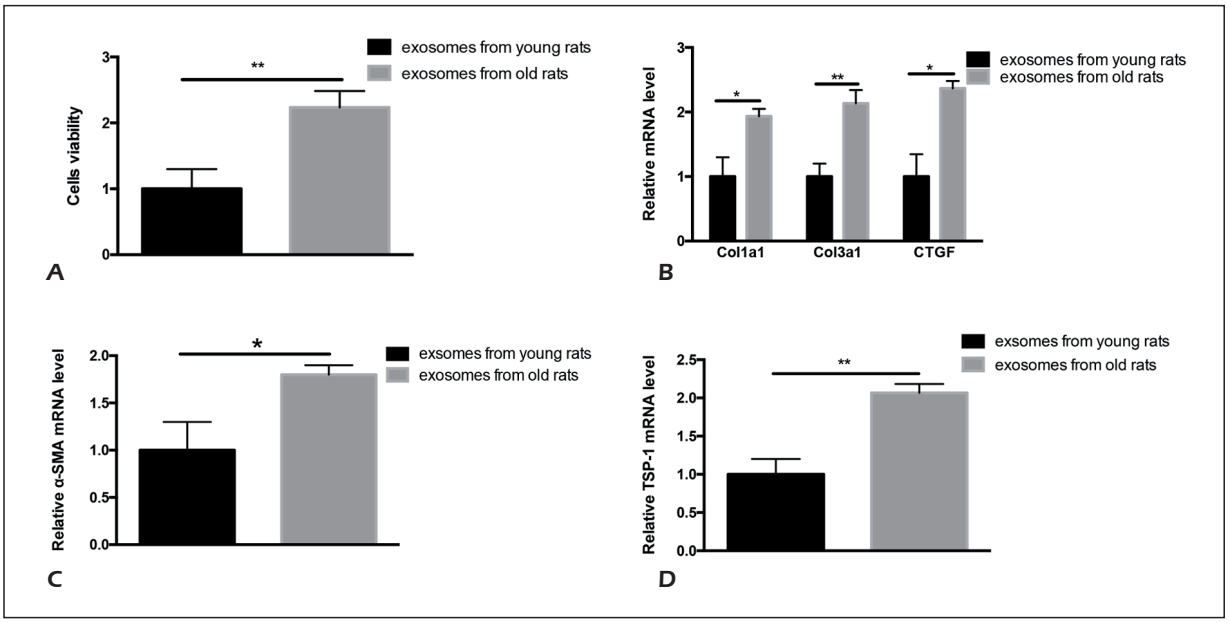

Figure 3. Exosomes from old rats promote fibroblast proliferation and myofibroblast differentiation. **A**, Cardiac fibroblast viability after a 24-h co-culture with exosomes from young or old rats. **B**, Comparison of Col1a1, Col3a1, and CTGF mRNA expression in cardiac fibroblasts after co-culture with exosomes from young or old rats. **C-D**, Comparison of α-SMA and TSP-1 expression in cardiac fibroblasts after co-culture with exosomes from young or old rats. α-SMA, alpha-smooth muscle actin; TSP-1, thrombospondin-1; Col1a1, collagen type 1 alpha 1; Col3a1 collagen type 3 alpha 1; CTGF, connective tissue growth factor; *p<0.05, **p<0.01.

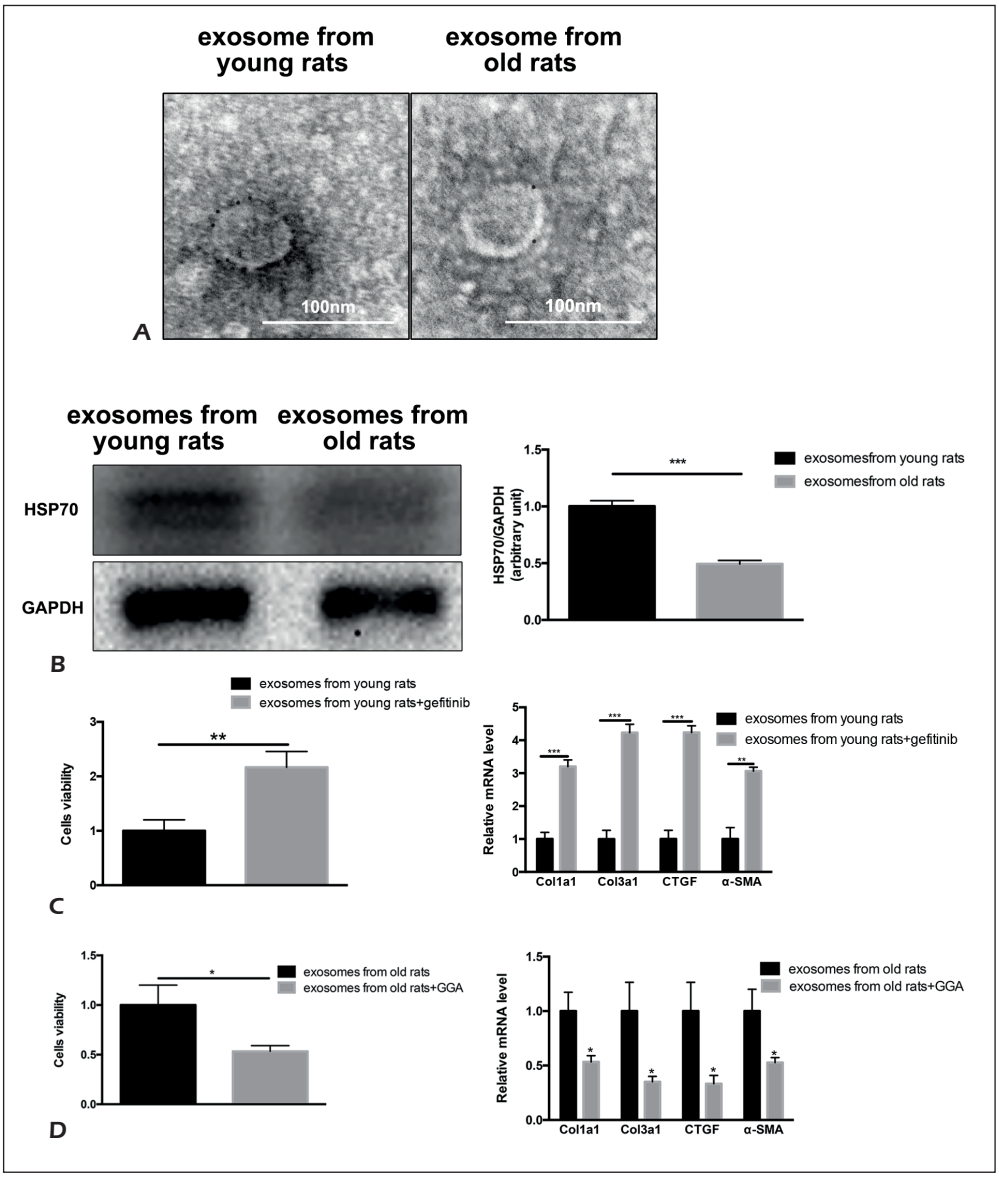

Figure 4. The role of exosomal HSP70 in senescence. **A**, Electron microscopic examination of surface HSP70 expression on exosomes from young or old rats. Scale bar, 100 nm. **B**, Western blotting analysis of HSP70 expression on exosomes from young or old rats. **C**, Examination of cell viability and Col1a1, Col3a1, CTGF, and α-SMA expression in cardiac fibroblasts following co-culture of cardiac fibroblasts with exosomes from young rats in the presence of gefitinib. **D**, Examination of cell viability and Col1a1, Col3a1, CTGF, and α-SMA expression in cardiac fibroblasts following co-culture of cardiac fibroblasts with exosomes from old rats in the presence of GGA. α-SMA, alpha-smooth muscle actin; Col1a1, collagen type 1 alpha 1; Col3a1 collagen type 3 alpha 1; CTGF, connective tissue growth factor; GGA, geranylgeranylacetone *p<0.05, **p<0.01, ***p<0.001.

scrutiny. This study shows that cardiac fibrosis, a process of adverse cardiac remodeling that can occur after MI or hemodynamic stress, is increased during aging. We show that the plasma levels of exosomes are increased during senescence, and that exosomes isolated from the plasma of aged rats promote cardiac fibrosis in an HSP70-dependant manner. We finally demonstrate a protective role for HSP70, where loss of HSP70 expression on the surface of exosomes contributes to an enhanced ability to promote fibrosis. These findings provide valuable insights into the processes involved in cardiac aging.

This study adds to the growing body of evidence suggesting that exosomes influence the fibrotic process[20-22]. Exosomes are natural transport nanovesicles (30-200 nm) secreted by numerous cell types. They originate from endosomes, are se-

creted from the plasma membrane, and play highly specific roles in the transport of DNAs, RNAs, or proteins. Intercellular transfer of exosomes is a well-established mechanism of cell-cell communication[27,28]. However, only a few studies have examined the effects of serum exosomes.

We showed that serum exosomes from old rats promoted the proliferation of fibroblasts and increased expression of Col1a1, Col3a1, CTGF, and α-SMA, indicating that during aging, plasma exosomes promote fibrosis. Our findings suggest that aging produces changes in the exosomes, one of which is a decrease in the expression of HSP70. Similarly, other studies have demonstrated a protective role of HSP70 against fibrogenesis. In 2007, Wakisaka et al[29] demonstrated both *in vitro* and *in vivo* that heat shock prevented the angiotensin-II-induced cardiac fibrotic response in the atrium, through the upregulation of HSP70. Angiotensin-II activates the MEK-ERK cascade, promoting atrial fibrosis[30] though the activation of the extracellular signal-regulated kinases (ERK)-activating kinases (MEK1/2), which activate ERK1/ERK2[31]. Heat shock-induced HSP70 upregulation attenuated the angiotensin-II-induced signals, thereby reducing ERK1/ERK2 phosphorylation and inhibiting the differentiation of fibroblasts into myofibroblasts by preventing α-SMA expression, TGF-β1 secretion, and extracellular matrix synthesis[29]. HSP upregulation has been proposed as a novel therapeutic approach to prevent atrial fibrosis[32].

In vivo, Tanaka et al[33] demonstrated that transgenic overexpression of HSP70 (tgHSP70) protected mice from protected from lung inflammation and bleomycin-induced fibrosis. The authors showed that the bleomycin-induced TGF-β1 production and pro-inflammatory cytokine expression was lower in tgHSP70 mice compared to wild-type mice. *In vitro*, HSP70 inhibition favored the epithelialtomesenchymal transition (EMT) process induced by TGF-β1 but, did not activate fibroblasts. The same team showed that administration of gefitinib, an HSP70 inhibitor, in mice exacerbated the pulmonary fibrosis induced by bleomycin[34].

In a model of kidney fibrosis, Zhou et al[35] proposed a mechanism of action for HSP70 in the TGF-β1 pathway that explained the protective effect of this HSP in fibrogenesis. They showed that HSP70 induction via GGA *in vivo* and *in vitro* inhibited phosphorylation and nuclear translocation of Smad3, thereby abrogating EMT and fibrosis. The authors further showed that the peptide-binding domain (PDB) of HSP70 was required for this protective effect, because overexpression of a mutant HSP70 lacking the PBD was unable to prevent EMT and fibrosis in their model. This study suggests that HSP70 acts as a chaperone of Smad3 via the PBD and sequesters it in the cytoplasm, thus blocking the TGF-β1-mediated activation of fibrogenesis[35]. Another study revealed a similar effect of HSP70 on the phosphorylation and nuclear translocation of Smad2 using a kidney cell line[36].

GGA, which is currently licensed to treat gastric ulcers, has been shown in animal studies to induce HSP70 expression *in vitro* and *in vivo,* and ameliorate fibrosis in several organs, including the lung and kidney[33,35,37]. This drug has been shown to prevent key events in the fibrotic process, including myofibroblast differentiation and EMT[33,37] GGA administration reduces organ damage in animal models of lung or kidney damage. In agreement with these findings, our study shows that the addition to GGA to *in vitro* cultures of exosomes and CFs reduced CF proliferation and myofibroblast differentiation.

Conclusions

We showed for the first time, that aging may be associated with an increase in exosomes production in the plasma. Exosomes from old rats were found to promote fibroblast proliferation and myofibroblast differentiation *in vitro*, and this enhanced ability was linked to the loss of HSP70 on their surface. This study provides valuable insights into how aging may act as a risk factor for cardiovascular disease.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1) Vigen R, Maddox TM, Allen LA. Aging of the United States population: impact on heart failure. Curr Heart Fail Rep 2012; 9: 369-374.
2) Bao Q, Pan J, Qi H, Wang L, Qian H, Jiang F, Shao Z, Xu F, Tao Z, Ma Q, Nelson P, Hu X. Aging and age-related diseases--from endocrine therapy to target therapy. Mol Cell Endocrinol 2014; 394: 115-118.

3) Noren Hooten N, Fitzpatrick M, Wood WH, 3rd, De S, Ejiogu N, Zhang Y, Mattison JA, Becker KG, Zonderman AB, Evans MK. Age-related changes in microRNA levels in serum. Aging 2013; 5: 725-740.
4) Childs BG, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. Nat Med 2015; 21: 1424-1435.
5) Lakatta EG. Cardiovascular aging research: the next horizons. J Am Geriatr Soc 1999; 47: 613-625.
6) Yang X, Sreejayan N, Ren J. Views from within and beyond: narratives of cardiac contractile dysfunction under senescence. Endocrine 2005; 26: 127-137.
7) Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics 2009; 6: 267-283.
8) Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, Hivroz C. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol 2002; 168: 3235-3241.
9) Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell 1983; 33: 967-978.
10) Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. J Exp Med 1996; 183: 1161-1172.
11) Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. Mol Biol Cell 1997; 8: 2631-2645.
12) Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. J Cell Biol 1999; 147: 599-610.
13) van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology 2001; 121: 337-349.
14) Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, Tursz T, Amigorena S, Raposo G, Angevin E, Zitvogel L. Malignant effusions and immunogenic tumour-derived exosomes. Lancet (London, England) 2002; 360: 295-305.
15) Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, Hager HD, Abdel-Bakky MS, Gutwein P, Altevogt P. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int 2007; 72: 1095-1102.
16) Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, Perdicchio M, Marino ML, Federici C, Iessi E, Brambilla D, Venturi G, Lozupone F, Santinami M, Huber V, Maio M, Rivoltini L, Fais S. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One 2009; 4: e5219.
17) Michael A, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, Alevizos I. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 2010; 16: 34-38.
18) Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A 2004; 101: 13368-13373.
19) Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer 2009; 10: 42-46.
20) Wang B, Yao K, Huuskes BM, Shen HH, Zhuang J, Godson C, Brennan EP, Wilkinson-Berka JL, Wise AF, Ricardo SD. Mesenchymal stem cells deliver exogenous microRNA-let7c via exosomes to attenuate renal fibrosis. Mol Ther 2016; 24: 1290-1301.
21) Zhao B, Zhang Y, Han S, Zhang W, Zhou Q, Guan H, Liu J, Shi J, Su L, Hu D. Exosomes derived from human amniotic epithelial cells accelerate wound healing and inhibit scar formation. J Mol Histol 2017; 48: 121-132.
22) Fang S, Xu C, Zhang Y, Xue C, Yang C, Bi H, Qian X, Wu M, Ji K, Zhao Y, Wang Y, Liu H, Xing X. Umbilical cord-derived mesenchymal stem cell-derived exosomal microRNAs suppress myofibroblast differentiation by inhibiting the transforming growth factor-beta/SMAD2 pathway during wound healing. Stem Cells Transl Med 2016; 5: 1425-1439.
23) Pagano F, Angelini F, Castaldo C, Picchio V, Messina E, Sciarretta S, Maiello C, Biondi-Zoccai G, Frati G, Meglio FD, Nurzynska D, Chimenti I. Normal versus pathological cardiac fibroblast-derived extracellular matrix differentially modulates cardiosphere-derived cell paracrine properties and commitment. Stem Cells Int 2017; 2017: 7396462.
24) Malik ZA, Kott KS, Poe AJ, Kuo T, Chen L, Ferrara KW, Knowlton AA. Cardiac myocyte exosomes: stability, HSP60, and proteomics. Am J Physiol Heart Circ Physiol 2013; 304: H954-965.
25) Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006; Chapter 3: Unit 3 22.
26) Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. Int J Mol Sci 2014; 15: 4142-4157.
27) Mittelbrunn M, Sanchez-Madrid F. Intercellular communication: diverse structures for exchange of genetic information. Nat Rev Mol Cell Biol 2012; 13: 328-335.
28) Lu A, Pfeffer SR. A CULLINary ride across the secretory pathway: more than just secretion. Trends Cell Biol 2014; 24: 389-399.
29) Wakisaka O, Takahashi N, Shinohara T, Ooie T, Nakagawa M, Yonemochi H, Hara M, Shimada T, Saikawa T, Yoshimatsu H. Hyperthermia treatment prevents angiotensin II-mediated atrial fibrosis and fibrillation via induction of heat-shock protein 72. J Mol Cell Cardiol 2007; 43: 616-626.
30) Booz GW, Carl LL, Baker KM. Amplification of angiotensin II signaling in cardiac myocytes by adenovirus-mediated overexpression of the AT1 receptor. Ann N Y Acad Sci 1999; 874: 20-26.

31) Goette A, Staack T, Rocken C, Arndt M, Geller JC, Huth C, Ansorge S, Klein HU, Lendeckel U. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. J Am Coll Cardiol 2000; 35: 1669-1677.
32) Takahashi N, Kume O, Wakisaka O, Fukunaga N, Teshima Y, Hara M, Saikawa T. Novel strategy to prevent atrial fibrosis and fibrillation. Circ J 2012; 76: 2318-2326.
33) Tanaka K, Tanaka Y, Namba T, Azuma A, Mizushima T. Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice. Biochem Pharmacol 2010; 80: 920-931.
34) Namba T, Tanaka K, Hoshino T, Azuma A, Mizushima T. Suppression of expression of heat shock protein 70 by gefitinib and its contribution to pulmonary fibrosis. PLoS One 2011; 6: e27296.
35) Zhou Y, Mao H, Li S, Cao S, Li Z, Zhuang S, Fan J, Dong X, Borkan SC, Wang Y, Yu X. HSP72 inhibits Smad3 activation and nuclear translocation in renal epithelial-to-mesenchymal transition. J Am Soc Nephrol 2010; 21: 598-609.
36) Li Y, Kang X, Wang Q. HSP70 decreases receptor-dependent phosphorylation of Smad2 and blocks TGF-beta-induced epithelial-mesenchymal transition. J Genet Genomics 2011; 38: 111-116.
37) Fujibayashi T, Hashimoto N, Jijiwa M, Hasegawa Y, Kojima T, Ishiguro N. Protective effect of geranylgeranylacetone, an inducer of heat shock protein 70, against drug-induced lung injury/fibrosis in an animal model. BMC Pulm Med 2009; 9: 45.